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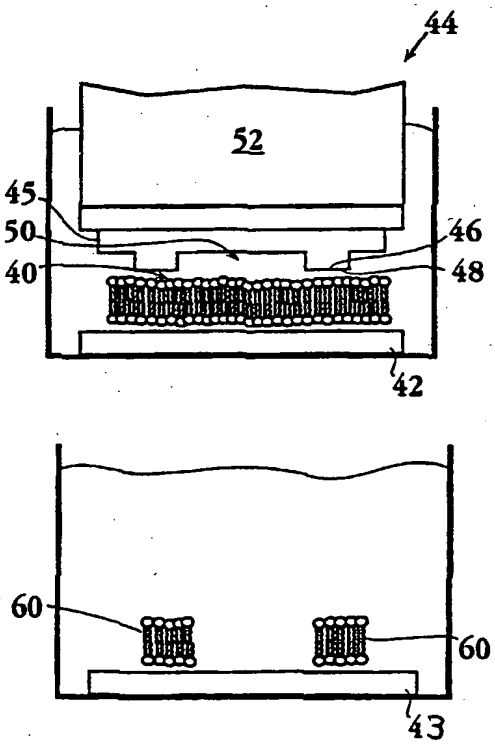

(57) Abstract: Methods for forming arrays of discreet, separated lipid bilayer regions on a substrate are disclosed. The methods exploit self-limiting diffusion of lipid bilayer regions on a substrate. Also disclosed are arrays formed by the methods, and uses of the arrays.

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LIPID-BILAYER ARRAYS AND METHODS OF MAKING AND USING SAME

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Field Of The Invention

The present invention relates to arrays of separated or discreet lipid-bilayer regions on a substrate, and to methods of making and using such arrays.

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Background Of The Invention

The ability to both functionalize and pattern surfaces is of widespread interest. Lipid bilayers on solid supports are especially challenging because they are two-dimensional fluids. When bilayers are assembled on glass supports, they are cushioned by a thin (5 - 20 Å) layer of water, so that both leaflets retain the fluidity that is an essential feature of biological membranes. The result is that the components are continually mixing and are free to diffuse across the entire surface. Such supported membranes retain many of the physical properties of natural cell membranes, and they can interact with living cells if the necessary

components are present. Thus, the functionalization and patterning of fluid supported lipid bilayers has applications in the fabrication of biosensors to achieve separations, for fundamental studies of membrane biophysics, and to construct an interface between hard surfaces and living cells.

One of the present inventors has previously reported methods for partitioning discreet regions of supported bilayers on a planar substrate surface. In one earlier-reported method, a supported bilayer expanse is partitioned by scratching the surface of a previously assembled continuous bilayer. The scratches function as barrier to lateral diffusion by a combination of topographical and tribological interactions. A second method involves patterning the properties of the solid support using photo- or electron beam lithography before the membrane is assembled. Depending on the chemical composition of the patterned material, lipids either do not assemble on the patterned regions or they assemble but are immobile.

In either case, the effect of patterning is to confine diffusive mixing to the corralled regions. Scratching is a simple method to apply, but it is not well controlled or entirely understood. Surface patterning requires the application of a second material to the surface, and the interaction of the lipids with the materials used is poorly understood. In both cases, the glass support must be chemically and/or physically altered.

Summary Of The Invention

The present invention includes, in one aspect, a method of forming a pattern of separated lipid regions on a substrate. The method includes the steps of forming over a planar portion of a substrate, a lipid-bilayer expanse sandwiched between a lower aqueous film and an upper aqueous bulk phase. A blotter having an embossed pattern of surface projections that (i) have contact surfaces capable of

supporting lipid-bilayer formation thereon, and (ii) form separated regions bounded by such surfaces, is applied to the bilayer expanse, serving to transfer regions of the lipid-bilayer expanse on the substrate to the blotter's contact surfaces. Removing the blotter leaves a pattern of separated lipid regions corresponding to the separated regions formed by the blotter projections.

In another aspect, the blotter to which portions of the lipid bilayer have been transferred is used to stamp a planar substrate covered by an aqueous medium, thereby to deposit on the substrate surface, a pattern of lipid bilayer regions corresponding to the pattern of surface projections in the blotter.

In both methods, the first substrates are formed of a material suitable for lipid-bilayer formation over an aqueous film, such as SiO_2, MgF_2, CaF_2, or mica. The blotter is formed of a material capable of picking up a lipid-bilayer, such as polydimethyl siloxane (PDMS), or surface-oxidized PDMS.

In a related aspect, the blotter to which portions of the lipid bilayer have been transferred is used to stamp a print medium, thereby to transfer the lipid-bilayer pattern to the print medium. The medium may be further developed to produce a colored pattern where the lipids were deposited.

Also disclosed is a surface-patterned device composed of a substrate having a planar surface, and formed on this surface, a pattern of lipid-bilayer regions sandwiched between a lower aqueous film and an upper aqueous bulk phase. The lipid regions are stably separated from one another by self-limiting lateral diffusion, without physical barriers between the regions on the substrate surface.

The substrate is formed of a suitable material for supporting lipid-bilayer regions over an aqueous film, such as SiO_2, MgF_2, CaF_2, or mica.

The lipid bilayer is formed of one or bilayer-forming lipids, such as phosphatidylcholine,

phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, and sphingomyelin, and optionally, bilayer-compatible lipids such as sterols and fatty acids. The device may contain 10^3 or more discrete bilayer-compatible surface regions, and the regions may be separated from one another by distances between 1 μm and about 10 μm.

The device may be designed for use in detecting binding events between one or more analytes and one or more selected lipid-bilayer-anchored biomolecules. Exemplary biomoelcules are transmembrane receptors or ion channels. Where the biomolecules include polynucleotides, the regions are in the form of an array of discreet regions, each carrying a different polynucleotide at a different region.

Also forming part of the invention is a method for use in detecting binding events between one or more analytes and one or more selected lipid-bilayer-anchored biomolecules. The method includes the steps of contacting a mixture containing such analyte(s) with a surface detector array device of the type just described and detecting binding of the selected ligand to receptors which specifically bind it.

In still another aspect, the invention includes a microfabrication process for producing a selected pattern on the surface of a substrate. The method includes the steps of forming, over a planar portion of such a substrate, a pattern of lipid-bilayer regions sandwiched between a lower aqueous film and an upper aqueous bulk phase. The pattern of lipid-bilayer regions corresponds to the selected pattern, and the lipid-bilayer regions are stably separated from one another by self-limiting lateral diffusion, without physical barriers between the regions on the substrate surface. Once the pattern of lipid-bilayer regions is formed on the substrate, the substrate is further processed to achieve the desired selected pattern.

In another aspect, the invention includes a method of forming an array of separated lipid regions on a substrate, by the steps of forming lipid-bilayer surface regions on the coplanar surfaces of a blotter of the type described above, and the transferring the lipid regions to a substrate having bilayer-compatible substrate regions separated by barriers. The transferring is effective to partially fill the substrate regions with lipid bilayers. Additional bilayer-forming lipids are then added to the substrate regions, to fill each substrate surface region with a lipid bilayer.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying drawings.

Brief Description Of The Figures

Fig. 1 shows a portion of a patterned substrate device formed in accordance with one aspect of the invention;

Figs. 2A through 2H are schematic illustration of two methods used to create patterned bilayer and bilayer-free regions on a substrate, in accordance with the invention, where Fig. 2A illustrates patterning by blotting and Fig. 2B, patterning by stamping;

Figs 3A through 3D are epifluorescence images of a supported lipid bilayer formed by embossing, shown after waiting for 30 minutes for self-limiting lateral expansion to occur into the regions where lipid was removed (Fig. 3A), and after an electric field (11V/cm and 1μA) was applied parallel to the bilayer plane for 65 minutes creating a steady-state gradient in the concentration of the negatively-charged Texas-red labeled lipid component (Fig. 3B);

Figures 5A and 5B depict partial-fill and in-fill perspective views.

Figs 4A and 4B are epifluorescence images of a supported lipid bilayer formed by stamping, where the image was taken immediately after photobleaching (Fig. 4A), and 46 minutes after photobleaching (Fig. 4B) showing partial recovery of the bleach as a result of lateral diffusion within the printed line; and

Figs. 6A through 6C depict steps in the preparation of a patterned substrate device constructed in accordance with another embodiment of the invention.

Figs. 7A through 7C represent a top down view of the bilayer arrays resulting from the practice of the present invention, for example as depicted in figs. 6A-6C.

Detailed Description Of The Invention

I. Definitions

The terms below have the following meanings unless indicated otherwise.

The term "aqueous" refers to a water-based liquid medium that is not deleterious to lipids.

The term "aqueous thin film" refers to a film of aqueous medium, typically 5-20 angstroms, and preferably about 10 angstroms, between a substrate surface and a lipid-bilayer region;

The term "aqueous bulk phase" refers to the layer of aqueous medium covering the lipid-bilayer regions on a substrate and extending into the lateral spaces between separated lipid-bilayer regions.

A surface "expanse" of lipid-bilayer refers to a substantially uninterrupted planar expanse of lipid-bilayer film on the surface of a substrate. The "expanse" is partitioned into a plurality of separated lipid-bilayer "regions" in accordance with the invention.

A "receptor" is a macromolecule capable of specifically interacting with a ligand or analyte molecule. In cells, receptors are typically associated with lipid bilayer

membranes, such as the extracellular, golgi or nuclear membranes. Receptors for incorporation into expanses of lipids *in vitro* (*e.g.*, supported bilayers) may either be purified from cells, recombinantly expressed, or, in the case of small receptors, chemically synthesized.

A "ligand" or "analyte" is a molecule capable of specifically binding to a receptor. Binding of the ligand to the receptor is typically characterized by a high binding affinity, *i.e.*, $Km>10^5$, and can be detected either as a change in the receptor's function (*e.g.*, the opening of an ion channel associated with or part of the receptor) or as a change in the immediate environment of the receptor (*e.g.*, detection of binding by surface plasmon resonance). Ligands for incorporation into expanses of lipids *in vitro* (*e.g.*, supported bilayers) may either be purified from cells, recombinantly expressed, or, in the case of small ligands, chemically synthesized.

Binding is "specific" if it results from a molecular interaction between a binding site on a receptor and a ligand, rather than from "non-specific" sticking of one protein to another. In cases where the ligand binds the receptor in a reversible manner, specificity of binding can be confirmed by competing off labeled ligand with an excess of unlabeled ligand according to known methods. Non-specific interactions can be minimized by including an excess of a protein (*e.g.*, BSA) that does not have binding sites for either the ligand or receptor.

II. Surface-Patterned Device

The present invention exploits the discovery herein that lipid-bilayers can form stable, self-limiting monolayers on a substrate surface, in effect, limiting the size of separated lipid-bilayer regions by the lateral diffusion. Membrane-forming lipids, when applied to a substrate surface, can assemble into bilayers as a result of a balance of

hydrophobic interactions, interfacial surface tension and repulsive intermolecular interactions; the interaction between planar bilayers and oxide supports involves a balance of van der Waals, electrostatic, hydration, and steric forces. As a consequence of the balance among these forces, supported lipid bilayers can expand only over a limited range on the surface. Lateral expansion does depend on pH: at high pH expansion is arrested, while at low pH it is initially quite rapid but then slows down over the course of a few minutes. The pH effect is likely related to the protonation state of the substrate, *e.g.*, glass surface and the resulting water structure at the interface.

Expansion is ultimately self-limiting because the lipids lose favorable interactions with each other. In one aspect, the invention exploits this self-limiting lateral expansion by gently removing bilayer material using a patterned polymer stamp. Once material is removed, the remaining supported membrane expands laterally, but the expansion halts leaving an essentially free-standing, but bounded, stable, and fluid region of bilayer material. Remarkably, it also proves possible to transfer the material that is removed onto a fresh surface, thereby stamping fluid bilayers in any desired pattern.

Fig. 1 is a perspective view of a portion of a surface-patterned device 20 constructed in accordance with the invention. The device is fabricated from a substrate 22 whose surface 24 is formed of a material such as an oxidized silicon or fused silica wafer. Exemplary dimensions of the substrate are typically between about 0.5 cm to about 5 cm per side and about 0.1 mm to about 1 cm in thickness.

Carried on the substrate surface are a plurality of separated lipid-bilayer regions, such as regions 26, 28, separated by channels, such as channel 30. The separated regions may be completely separated from one another, *e.g.*, completely surrounded by separating channels, or adjacent

regions may be joined by lipid-bilayer "bridges" that may allow lipid diffusion between joined regions, but which are stably formed by virtue of self-limiting diffusion of the lipids making up the bridge.

Interposed between each lipid-bilayer region and the underlying region of the substrate surface is an aqueous film 32 that is between about 5 Å and 20 Å (typically about 10 Å) in thickness. The degree of "bilayer-compatibility" of a selected surface is a function of its intrinsic material properties rather than its shape. The interactions between lipid-bilayers and a substrate involve electrostatic and hydration forces as well as attractive contributions from long-range van der Waals forces. These forces act to stabilize the single lipid bilayer, and to anchor the bilayer region stably to the surface, to prevent the regions from migrating laterally on the substrate surface. In a suitable bilayer-compatible surface, an energetic minimum traps the bilayer membrane between about 5 Å and 20 Å (typically about 10 Å) away from the supporting surface, separated from the supporting surface by an aqueous film of corresponding thickness. Covering the lipid-bilayer and the channels separating these regions is a bulk aqueous phase 34.

Bilayer-compatible surfaces are typically hydrophilic. It will be appreciated that many materials suitable for use in the microfabrication of a device according to the invention will, when cleaned, present a bilayer-compatible surface region. Exemplary materials having properties making them suitable for bilayer-compatible surfaces include various glasses, silicon oxides, including oxidized silicon (SiO_2), MgF_2, CaF_2, mica, and various polymer films, such as thin polyacrylamide or dextran films (see, *e.g.*, 23; 24), both incorporated herein by reference). Both types of polymer films form a suitable bilayer-compatible surface that is hydrated to provide a film of aqueous between the polymer film and the supported bilayer membrane. Additional details

of suitable bilayer-compatible surfaces, and bilayer-barrier regions are detailed in co-owned U.S. patent application SN 08,978,756, filed November 26, 1997, which is incorporated herein.

The supported bilayer itself is a self-assembling, two-dimensional fluid system, typically consisting of two opposed leaflets of vesicle-forming lipid molecules. However, it can be constructed as described below from any suitable membrane-forming amphiphile. Most vesicle-forming lipids are long-chain carboxylic acids, such as glycerides, having the hydroxyl groups of the glycerol esterified with (i) fatty acid chain(s), and (ii) a charged or polar moiety, such as a phosphate-ester group. The vesicle-forming lipids are preferably ones having two hydrocarbon chains, typically acyl chains, and a polar head group. Long-chain carboxylic acids with a phosphate group, or phospholipids, are particularly well-suited for use with the present invention.

There are a variety of synthetic vesicle-forming lipids and naturally-occurring vesicle-forming lipids, including the phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid, phosphatidylinositol (PI), phosphatidylglycerol (PG), and sphingomyelin, where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. The above-described lipids and phospholipids whose acyl chains have varying degrees of saturation can be obtained commercially or prepared according to published methods. Other suitable lipids include glycolipids and sterols such as cholesterol.

Preferred diacyl-chain lipids for use in the present invention include diacyl glycerol, phosphatidyl ethanolamine (PE) and phosphatidylglycerol (PG). These lipids are preferred for use as the vesicle-forming lipid, the major

liposome component, and for use in the derivatized lipid described below.

All of these phospholipids and others are available from specialized suppliers of phospholipids (*e.g.*, Avanti Polar Lipids, Inc., Alabaster, Alabama) as well as from general chemical suppliers, such as Sigma Chemical Co. (St. Louis, MO).

The aqueous film and bulk aqueous phase may be any suitable aqueous solution, such as a buffered saline solution (*e.g.*, PBS). The bulk solution can be readily changed (taking care, of course, to keep the supported bilayer submerged at all times) by, *e.g.*, flow-through rinsing with a solution having a different composition.

A number of different-dimension arrays of lipid regions may be produced in accordance with the invention. They include, as example, (i) a device containing a 1 cm^2 array of 2500 identical 200 μm square corrals or regions, (ii) a device containing a 1 cm^2 array of 10,000 identical 100 μm square regions, (iii) a device containing a 1 cm^2 array of about 37,000 identical 50 μm square regions separated by 2 μm channels, and (iv) a device containing a 1 cm^2 array of about 2.8 million 5 μm square corrals or regions separated by 1 μm-wide channels.

Exemplary embodiments of the invention include devices where the bilayer lipid expanses contain different biomolecules, such as receptor protein molecules, ligand protein molecules, or other protein molecules. Such devices are particularly useful in biosensors, described more fully in the applications section of the specification, and are made as described below by fusing proteoliposomes to the bilayer-compatible surface.

It is recognized that proteoliposome vesicles can be fused to a glass surface to create a planar supported membrane (5). This technique has been successfully applied in a number of situations. In one example, the H2Kk protein

was reconstituted into egg phosphatidylcholine- cholesterol vesicles by detergent dialysis, and the vesicles were used to create a planar membrane on glass (5). The H2Kk-containing membrane was capable of eliciting a specific cytotoxic response when brought into contact with a cell.

Chan, *et al.* (22) demonstrated that a glycosylphosphatidylinositol (GPI)-anchored membrane receptor is laterally mobile in planar membranes formed from proteoliposome fusion, and that this mobility enhances cell adhesion to the membrane. Other applications employ a combination of vesicle fusion, Langmuir-Blodgett methodology and derivatized surfaces to prepare supported membranes.

In addition to incorporation of receptors or ion channels into the bilayer membrane, the bilayer may be derivatized with any of a number of groups or compounds to create a surface having the desired properties. For example, the liposomes may contain a ligand bound to the surface of the lipid by attachment to surface lipid components. Generally, such a ligand is coupled to the polar head group of a vesicle-forming lipid. Exemplary methods of achieving such coupling are described below.

It will be appreciated that the device is designed, *e.g.*, by microfabication, to include other elements needed for carrying out the intended function of the device. For example, in a device designed for use as a biosensor, the substrate regions supporting the separated lipid-bilayer regions may be electrodes, *e.g.*, connected to the substrate surface through a salt bridge, for measuring ion flow across the membrane regions, for example, where the membrane regions are constructed to contain ion channels. Alternatively, a device for measuring transmembrane currents may include pH sensitive dyes or the like that can be used to monitor ion flow across the membrane. In still another embodiment , the device is designed for measuring analyte binding to one or more different biomolecules incorporated into the separated

bilayer regions. Here the device may be used in combination with an optical scanning device for measuring binding of an optically active analyte to one or more regions in the device.

III. Construction of A Surface-Patterned Device

The invention contemplates three different methods for forming surface-patterned arrays of lipid-bilayers regions on a substrate.

A. Blotting The first of these methods is referred to as "blotting" and is illustrated in Fig. 2A. The method involves first forming a continuous lipid-bilayer expanse on a preferably planar substrate 42. Methods of forming the lipid expanse are detailed elsewhere, including references 10 and 14, and as detailed in co-owned US patent application SN 08/978,756.

As shown in Figure 2B, there is then applied to the expanse, a blotter 44 having an embossed pattern of surface projections, such as projection 46 that (i) have contact surfaces, such as surface 48, capable of supporting lipid-bilayer formation thereon, and (ii) form separated regions, such as region 50, bounded by such surfaces. The blotter is preferably formed of a polymeric material, and a preferred material is polydimethylsiloxane (PDMS), which may be surface oxidized, *e.g.*, by plasma treatment. The blotter also contains a weight 52 for holding the blotter against the substrate in a submerged condition.

According to an important aspect of the invention, this blotting is effective to transfer lipid-bilayers regions in contact with the contact surface48 to such surfaces, leaving channels, such as channels 54 between now-separated lipid-bilayer regions, such as regions 56, when the blotter is removed. Removed lipid bilayer 60 may then be transferred to a second glass surface 43, figure 2C, and deposited as shown in figure 2D.

In another preferred embodiment is shown in figures 2E through 2G, where bilayer material is pre-assembled from vesicles 61 to "ink" oxidized PDMS surfaces 48 that are then transferred to glass 42 by stamping to reliably transfer of membrane patches 60 to glass 42.

As one exemplary method, vesicles were prepared from egg phosphatidylcholine (egg PC) from Avanti Polar Lipids with 1 mol % N-(Texas Red sulfonyl)-1,2-dihexandeconoyl-sn-glycero-3-phosphoethanolamine (Texas Red DHPE) from Molecular Probes. The preparation of supported lipid bilayer from vesicles has been outlined in detail elsewhere. The pH of the lipid suspension was such that spreading is expected to occur. As a result it is necessary to chose the amount of material removed vs. amount of material left such that after expansion a bilayer-free region remains. A Nikon E800 fluorescence microscope equipped with a Photometrics Sensys CCD camera was used to image the bilayers. Electrophoresis within the membrane was performed using methods laid out previously, in Millipore water with currents < 2μA which produces a negligible amount of resistive heating.

Polydimethyl siloxane (PDMS) stamps were formed by curing Sylgard 184 (Dow Corning) on silicon masters with patterned photoresist. More preferably, Sylgard 182 may by used because it does not harden below 65°C, suggesting that the softness of the PDMS is important. The masters were created using Shipley 3612 positive photoresist, 1μm thick, on silicon wafers that were vapor primed with hexamethyldisilazane. The patterning was achieved using standard photolithographic techniques. The PDMS is a square grid 1μm high; the width of the grid lines is 15 μm and the lines are 215 μm apart. Using this stamp to emboss a lipid bilayer should yield grid lines that are about 3 μm wide and 227 μm apart.

Figure 3A depicts an epifluorescence image of a supported lipid bilayer after it was embossed for ten minutes in ultrapure water. The image was taken approximately 30 minutes after embossing, at which point the bilayer has essentially finished expanding into the region where the membrane was removed. A dark grid pattern is clearly visible in the image where the lipids have been removed, and the pattern was shown to be stable under water for at least one week. The expansion of the bilayer is responsible for the ragged edges of the pattern, as the expansion front of a bilayer proceeds in finger-like projections on glass surfaces. The fluorescence intensity in the grid (dark) areas is very close to background levels, indicating that lipid material has been nearly completely removed from the grid area, that is, both leaflets of the bilayer appear to be removed. The grid lines are approximately 19 μm wide rather than the expected 3 μm. However, as one of the properties of PDMS is that it is deformable, it would not be surprising if the area of the PDMS in contact with the bilayer during embossing was larger than expected.

To determine if this pattern functions as a barrier to lateral diffusion we applied an electric field parallel to the plane of the bilayer. Electrophoresis causes the negatively charged Texas Red-labeled lipids to be drawn towards the positive electrode, forming a gradient at steady state as they build up against the boundary of a patterned region. Figure 3B depicts an image of the same area as Figure 3A after an electric field of 11 V/cm and 1 (A was applied for 65 minutes. A concentration gradient of bright fluorescence is seen on the right side of each partitioned region demonstrating that the lipids are mobile within the square corrals but are confined. Upon removing the field, the lipids relax back to uniformity; the electric field direction can be reversed and the gradient forms along the

opposite side. Thus, it is seen that the bilayer-free regions created by removing lipids with the PDMS stamp serve as stable barriers to lateral diffusion. Fluorescence recovery after photobleaching (FRAP) experiments were also performed (data not shown), and these demonstrated recovery within the patterned regions but not across boundaries.

Figure 3E depicts a pattern made by depositing small bilayer regions onto surfaces, separated by relatively larger barrier regions. Figure 3F depicts bilayer regions surrounded by similarly sized barrier regions. Figures 3G through 3I depict photographs of fluorescent material migrating under the effect of an applied electric field across the pan-handle shaped bilayer region. This demonstrates that the shape of a bilayer region may be used in conjunction with external forces to concentrate, dilute, mix, or transfer materials.

An interesting situation arises at the outside borders of the embossed region. A stamp half the size of the bilayer-covered region was used, so that only half the bilayer area was patterned. Because the patterned areas at the borders were adjacent to a larger reservoir of material then those in the interior, as the bilayer expands the barriers at the edges should be and are erased. This result means that all that is necessary to create barriers to diffusion is to remove enough material so that after the subsequent expansion the bilayer regions are left unconnected. Additionally, it is possible to erase all of the barriers to diffusion by incubating with vesicles, *i.e.* adding material back in. After incubation the lipids are once again free to diffuse across the entire surface.

B. Stamping In this method, which is illustrated in Figs. 2A through 2D, lipid-bilayer regions are transferred from a first substrate covered with a planar lipid expanse, as above, to the contact surfaces of a blotter 44, to produce a pattern of bilayer regions, such as regions 60, on the

coplanar blotting surfaces of the blotter, as shown. The step of forming the bilayers on the blotter surfaces may be similar to the blotting method described above, where the blotter is applied to a planar lipid expanse on a substrate, or may be by direct formation of lipid bilayer regions on the blotter surfaces. In this second method, vesicles are placed in contact with the blotter surfaces under water, *e.g.*, for at least an hour, until a layer of lipid bilayer forms on the exposed surfaces. Excess vesicles are then removed, *e.g.*, by shaking them off the blotter.

In the final step of the method, shown in Fig. 2D, the blotter is stamped on second substrate 43, to transfer the lipid-bilayer regions on the blotter surfaces to the substrate.

Figure 4A shows epifluorescence images of the material that is stamped using the first inking procedure and a grid pattern that is identical to that shown in Figure 3A. The bright pattern of fluorescence corresponds to the region that was removed in Figure 3A, giving further evidence that the embossing method actually removes bilayer material. In order to test whether the transferred lipids are themselves assembled into a fluid bilayer, a region of the fluorescence just below the center (Figure 4A) was photobleached. Figure 4B is the same region 46 minutes later, and it is evident that much of the fluorescence in the bleached region has recovered. Close inspection indicates that recovery is not complete; we suspect that the removal and deposition processes as currently practiced leave some regions that are not fully covered, though sufficiently connected to permit long-range diffusion. The fluorescence level of the transferred lipids is approximately 60% that of the fluorescence level of the surface from which the lipids were removed; of the 40% lost approximately 10% was lost in the removal and 30% was lost in the stamping. We have done little to optimize this system, and variations in the

methods, the stamp material or the stamp topography may prove fruitful. Similar results were obtained with the second method. We note in passing that there is an inversion of the membrane leaflet that is in contact with the receiving surface in these two methods. In the first method, the side of the supported membrane originally in contact with the glass remains in contact after transfer and printing. In the second method, where the assembly is initially on the PDMS surface, the transfer should place the side that is in contact with the bulk solution when on PDMS in closest contact with the receiving glass surface. This may prove useful for inverting the orientation of self-assembled systems.

Patterns formed by stamping could also be erased by incubating with vesicles as was the case for patterns formed by embossing. The addition of more material to a surface that is clean except in the patterned region, is another demonstration that the patterns are maintained by the self-limiting lateral expansion of supported lipid bilayers.

C. Lipid-Region Augmentation A third method of forming lipid regions is illustrated in Figs. 5A, 5B, and 5C. In this method, discreet lipid regions are formed on the contact surfaces of a blotter, as above. These lipid regions are then transferred by stamping to corresponding regions of a substrate, such as substrate 70 in Figs. 5A, 5B, and 5C. Preferably, the substrate is one like that described in co-owned US patent application SN 08/978,756, having lipid-bilayer compatible surface regions, or lipid regions, such as regions 72, 74, separated by lipid-bilayer barriers, such as barrier 76. Contact surfaces 46a and 46b on blotter 46 preferably have a one-to-one correspondence with lipid regions72 and 74 on the substrate, and can be placed in registry therewith, such that each blotter contact surface deposits its lipid bilayer on a corresponding lipid region of the substrate.

As illustrated in Fig. 5A, the lipid transfer stamping is effective to partially fill each substrate lipid, producing islands of lipid bilayers within each region. According to one aspect of this method, the amount of lipid transferred to each of the substrate regions in the blotter-transfer step is selectively varied, by selectively varying the surface area of each of contact surface of the blotter. Thus, in Figs. 5A, it might be assumed that the four areas shown have increasing amount of lipid, in progressing in right-to-left and back-to-front directions. Figs. 7A shows a top down view of such distribution of different amounts of a first lipid material into different, spaced-apart locations. Fig. 6A depicts the "inked" stamped method disclosed above, resulting in Fig 6B patches 60 spatially distributed on surface 42 separated by barrier regions. In-fill is depicted in figure 6C by the addition of lipid vesicles 61 which contact patches 60 and/or surface 42 and fill in the bilayer region up to the point of the bilayer barriers. Referring back to Figures 7A through 7C, Fig. 7A is a top down view of the step depicted in figure 6B. Figures 7B represents a top down view of Fig. 6C, illuminating only lipid component attributable to patches 60. Figure 7C depicts the illumination of the in-fill lipid vesicle material. Thus, taken together, Figures 7A through 7C demonstrate how the instant invention provides methods and devices for creating arrays of independent, supported lipid bilayer regions, supported by barrier regions, wherein each bilayer region comprises a different lipid composition or ratio or lipids.

Following the transfer step, the substrate is exposed to lipid vesicles effective to form lipid-bilayer regions that "fill in" or complete the lipid coverage in each of the substrate's lipid regions as shown in Fig. 5B. Figs. 5e and 5f, under different illumination conditions, show the amounts of the first and second bilayer forming materials in each location. This can be done, for example, by exposing the

partially filled substrate to lipid vesicles, similar to the producing of planar expanses on the substrate as discussed above. With this filling in, each lipid region on the substrate, such as regions 72, 74, is covered with a lipid bilayer region having a defined ratio of lipid material deposited by blotter transfer to lipid material subsequently added.

The method is useful, for example, in producing arrays with different selected amount of two or more lipids, and/or different ratios of lipid/non-lipid biomolecules, *e.g.*, lipid/receptor protein. In the latter case, for example, a blotter having different-size contact surface areas might be prepared with a lipid-bilayer containing a fixed ratio of lipid/protein. When this material is transferred to the substrate, different selected amount of lipid and protein are transferred to the different-sized blotter surface regions, and thus different selected amounts of protein are transferred to the individual substrate lipid regions. Following lipid addition to the substrate, each region will have a different selected lipid/protein ratio.

Other methods of forming lipid-bilayer regions with selected ratios of components are also contemplated, in accordance with the invention. For example, a grid a small-sized lipid regions can be blotted to remove different-selected size interior regions. By filling in these interior regions with lipid vesicles or by stamping from a blotter, desired ratios of components can be deposited in each array region.

The invention has a variety of uses in microfabrication, and printing, as well as in diagnostic and drug-screening applications, as can be appreciated by those skilled in the art.

For example, in microfabrication, a pattern of lipid regions formed in accordance with the invention could be used to affix a non-lipid molecule, *e.g.*, a protein, *e.g.*, that

has a lipid-compatible moiety. After securing the biomolecule to the surface, *e.g.*, with a fixative, the original lipid pattern could be removed. Likewise, in printing, a pattern of lipid bilayer regions formed on a stamp can be transferred to a selected print medium, *e.g.*, paper, then subsequently used to capture a, lipid-compatible dye. After the dye is captured and affixed to the medium, the lipids may be removed, *e.g.*, by a wash step.

Experimental

Small unilamellar vesicles were prepared from egg phosphatidylcholine (egg PC, Avanti Polar Lipids) with 1 mol% Texas Red® 1,2-dihexadecanoyl-sn-glycero- 3-phosphoethanolamine, triethylammonium salt (Texas Red® DHPE, Molecular Probes) or 3 mol% Marina Blue® 1,2-dihexadecanoyl-sn-glycero- 3-phosphoethanolamine (Marina Blue® DHPE, Molecular Probes). The appropriate amounts of each kind of lipids were mixed together in chloroform, dried under N_2, and put under vacuum for at least 40 minutes. The lipids were then reconstituted with Millipore (18MΩ) water and were passed 19 times through an Avanti extruder containing a membrane with 50nm pores. The extruded vesicles were stored at 4°C and were used within 3 days. It is preferable to use 50nM extruded vesicles prepared less than three days prior to use, and more preferably, prepared just prior to use.

A Nikon E800 fluorescence microscope equipped with a Photometrics Sensys CCD camera was used to image the bilayers. To image the Texas Red fluorophores a Texas Red filter (Chroma Technology Corp.) set was used; Marina Blue fluorophore were imaged using a Cascade Blue filter set (Chroma Technology Corp.). The Cascade Blue filter set matches the spectrum of Marina Blue reasonably well. Electrophoresis within the membrane was performed, using methods laid out previously, in Millipore water with currents

< 2μA which produces a negligible amount of resistive heating. Diffusion coefficients of the fluorescent probes were determined by Fourier analysis of the time evolution of a fluorescence profile using a custom fitting program.

Polydimethyl siloxane (PDMS) stamps were formed by curing Sylgard 182 (Dow Corning) on silicon masters with patterned photoresist at 70°C for 80 minutes. The masters were created using Shipley 3612 negative photoresist, 1μm or 1.65μm thick, on silicon wafers, which were vapor primed with hexamethyldisilazane. The patterning was achieved using standard photolithographic techniques. After developing, the wafers were vapor primed again with hexamethyldisilazane to assist in the subsequent removal of the PDMS. Flat PDMS stamps were formed by curing on silicon wafers that were vapor primed with hexamethyldisilazane. Surface oxidation of PDMS was carried out using a plasma cleaner (Harrick Scientific) under high power for 15 to 60 seconds while a small amount of air was leaked into the chamber. Glass slides were prepared by washing in ICNx7 detergent (ICN, Costa Mesa, CA) followed by exhaustive rinsing in distilled water and then baking in a kiln at 400°C for at least 4 hours. It is preferable to use such prepared glass within three days of cleaning.

Microcontact printing of the lipid bilayers was carried out as follows. Within 30 minutes of oxidation the PDMS was brought into contact with a solution of lipid vesicles (either in water or mixed with 5mM Tris, 50mM NaCl, at pH 8 buffer) for 1 min, then the excess vesicle were washed away with large amounts of water. More preferably, a 10 mM Tris, 100 mM NaCl, pH 8.0 can be used instead of 18 meg-ohm ultrapure water. Keeping the PDMS in water at all times the "inked" PDMS was then brought into contact, using a light weight (5.2g), with a glass surface for preferably 10-15 seconds in a solution of 5mM Tris, 50mM NaCl, at pH 8.0.

Permanent grid patterns, which serve as barriers for membrane partitioning, were produced on glass by microcontact printing bovine serum albumin (BSA) as described in detail elsewhere.[9] In brief, a 20µl solution of 80µg/ml of Alexa488 labeled BSA (Molecular Probes) in a 17mM, pH 8.0 phosphate buffer was placed on an oxidized PDMS stamp, allowed to sit for 10min, the excess was shaken off, and then the stamp was dried under N_2. The stamp was brought into contact with a glass slide using a light weight (14g) for 30 seconds. The slide was rinsed vigorously in deionized water to remove the excess BSA and dried under N_2.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications may be made without departing from the invention.

IT IS CLAIMED:

1. A method of forming a pattern of separated lipid regions on a substrate, comprising

forming, over a planar portion of a substrate, a lipid-bilayer expanse sandwiched between a lower aqueous film and an upper aqueous bulk phase,

applying to the expanse, a blotter having an embossed pattern of surface projections that (i) have contact surfaces capable of supporting lipid-bilayer formation thereon, and (ii) form separated regions bounded by said contact surface,

by said applying, transferring regions of the lipid-bilayer expanse on the substrate that are in contact with said contact surfaces to the contact surfaces, leaving on the substrate, a pattern of separated lipid regions corresponding to the separated regions formed by the contact surfaces, when the blotter is removed.

2. The method of claim 1, wherein the substrate surface is formed of a material selected from the group consisting of SiO2, MgF2, CaF2, and mica.

3. The method of claim 1, wherein said contact surfaces are formed of PDMS or surface-treated PDMS.

4. A method of forming a pattern of separated lipid regions on a substrate, comprising

on a blotter having an embossed pattern of surface projections that define contact surfaces capable of supporting lipid-bilayer formation thereon,

forming lipid-bilayer regions on said contact surfaces, and

stamping a planar substrate covered by an aqueous medium with the blotter, thereby to transfer lipid bilayer regions on the contact surfaces to the substrate, to form on the

substrate, a pattern of lipid bilayer regions corresponding to the pattern of surface projections in the blotter.

5. The method of claim 4, wherein the first and second surfaces are formed of a material selected from the group consisting of SiO2, MgF2, CaF2, and mica.

6. The method of claim 4, wherein the blotter contact surfaces of formed of PDMS or surface-oxidized PDMS.

7. The method of claim 4, wherein said forming includes applying the blotter to a planar expanse of lipid bilayer contained on another substrate, to transfer bilayer regions from the substrate to the blotter's contact surfaces.

8. The method of claim 4, wherein said forming includes exposing the blotter's contact surfaces to a suspension of lipid vesicles, with the contact surfaces in a submerged state.

9. A surface-patterned device comprising

a substrate having a planar surface, and

formed on said surface, a pattern of lipid-bilayer regions sandwiched between a lower aqueous film and an upper aqueous bulk phase, said lipid regions being stably separated from one another by self-limiting lateral diffusion, without physical barriers between the regions on the substrate surface.

10. The device of claim 9, whose surface is formed of a material selected from the group consisting of SiO2, MgF2, CaF2, and mica.

11. The device of claim 9, wherein the lipid bilayer expanse comprises at least one lipid selected from the group

consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, and sphingomyelin.

12. The device of claim 11, wherein the lipid bilayer expanse is composed predominantly of phosphatidylcholine.

13. The device of claim 9, which contains at least 5 x 103 discrete bilayer-compatible surface regions.

14. The device of claim 9, wherein the lipid-bilayer regions are separated from one another by distances between 1 μm and about 10 μm.

15. The device of claim 9, for use in detecting binding events between one or more analytes and one or more selected lipid-bilayer-anchored biomolecules, which further includes such one or more biomolecules anchored in the lipid-bilayer regions.

16. The device of claim 15, wherein the one or more biomolecules include one or more transmembrane receptors or ion channels.

17. The device of claim 15, wherein the biomolecules include polynucleotides, and the regions are in the form of an array of discreet regions, each carrying a different polynucleotide at a different region.

18. A method for use in detecting binding events between one or more analytes and one or more selected lipid-bilayer-anchored biomolecules, comprising

(a) contacting a mixture containing such analyte(s) with a surface detector array device, said device comprising (i) substrate having a planar surface, and (ii) formed on said

surface, a pattern of lipid-bilayer regions that are sandwiched between a lower aqueous film and an upper aqueous bulk phase, said regions (i) being stably separated from one another by self-limiting lateral diffusion, without physical barriers between the regions on the substrate surface, and (ii) containing one or more of such biomolecules, and

(b) detecting binding of the selected ligand to receptors which specifically bind it.

19. The method of claim 18 for use in determining sequence information about a selected polynucleotide analyte or analytes, wherein the biomolecules are polynucleotides.

20. A microfabrication process for producing a selected pattern on the surface of a substrate, comprising

forming, over a planar portion of such a substrate, a pattern of lipid-bilayer regions sandwiched between a lower aqueous film and an upper aqueous bulk phase, said pattern corresponding to such selected pattern and said lipid-bilayer regions being stably separated from one another by self-limiting lateral diffusion, without physical barriers between the regions on the substrate surface, and

further processing the substrate to achieve the desired selected pattern.

21. A printing process for producing a selected pattern on the surface of a print medium, comprising

on a blotter having an embossed pattern of surface projections that define contact surfaces capable of supporting lipid-bilayer formation thereon,

forming lipid-bilayer regions on said contact surfaces,

stamping the blotter onto the print medium, thereby to transfer the lipid-bilayer regions on the blotter to the print medium, and

further processing the print medium to form a visible pattern related to the pattern of lipid-bilayer regions on the medium.

22. A method of forming an array of separated lipid regions on a substrate, comprising

on a blotter having an embossed pattern of surface projections that define contact surfaces capable of supporting lipid-bilayer formation thereon,

forming lipid-bilayer regions on said contact surfaces,

transferring the lipid-bilayer regions to a substrate having surface regions capable of supporting lipid-bilayers thereon, said regions being separated by barriers that limit the lateral expansion of lipid-bilayers in such regions,

where said transferrring is effective to partially fill the substrate regions with lipid bilayers on the blotter, and

adding additional bilayer-forming lipids to said substrate regions, thereby to fill each substrate surface region with a lipid bilayer.

23. The method of claim 22, wherein the lipid bilayer regions on the blotter contain known, selected amounts of one or more lipid-associated biomolecules, and said adding is effective to form on the substrate, separated lipid-bilayer regions with known ratios of lipid and lipid-associated biomolecules.

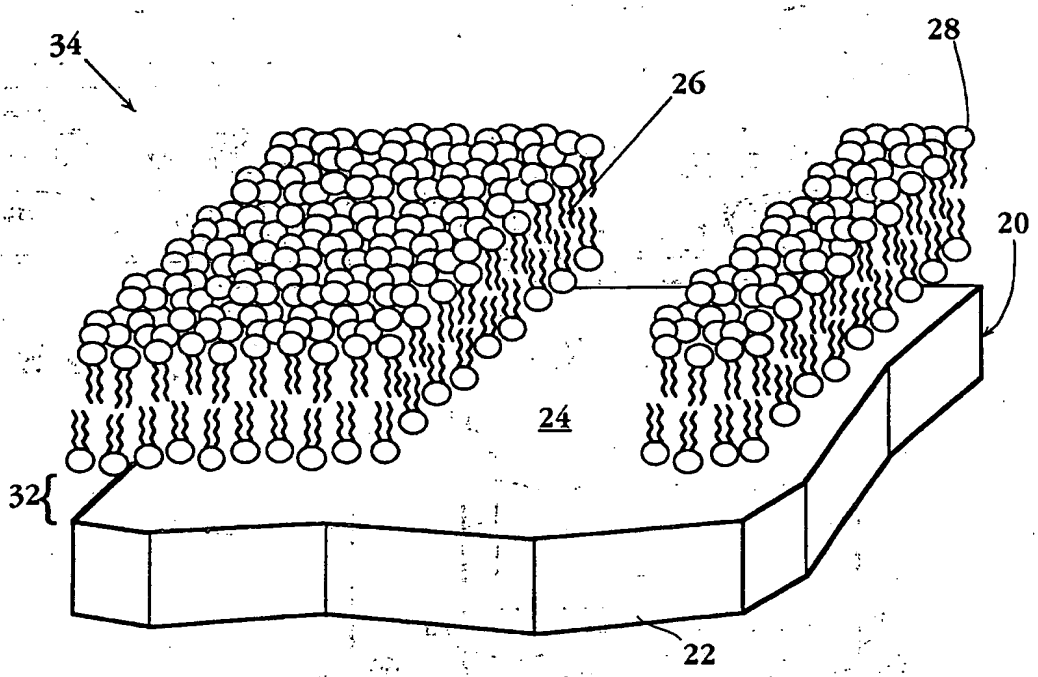

Fig. 1

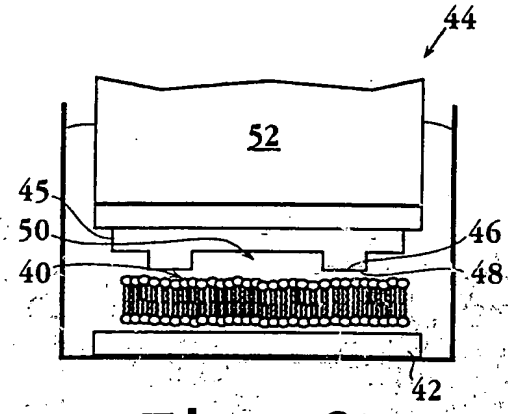

Fig. 2A

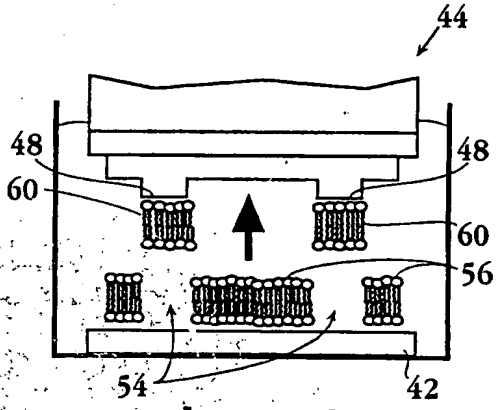

Fig. 2B

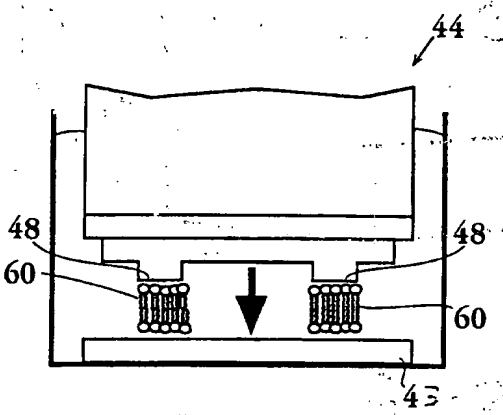

Fig. 2C

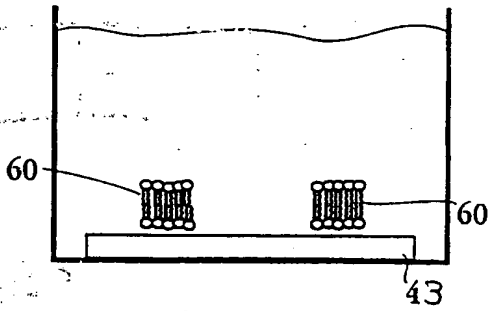

Fig. 2D

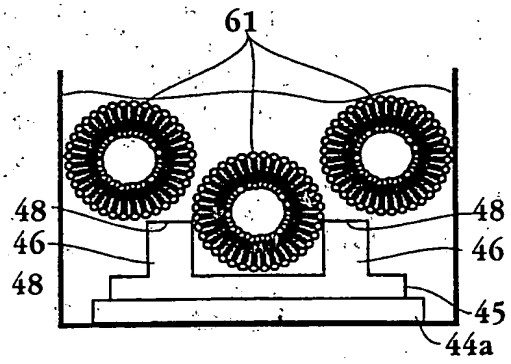

Fig. 2E

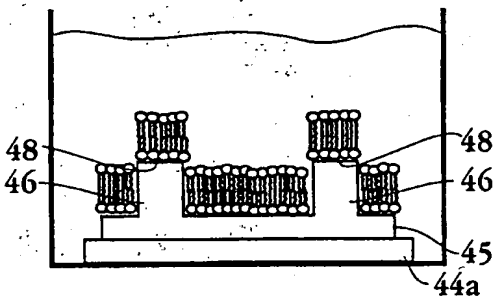

Fig. 2F

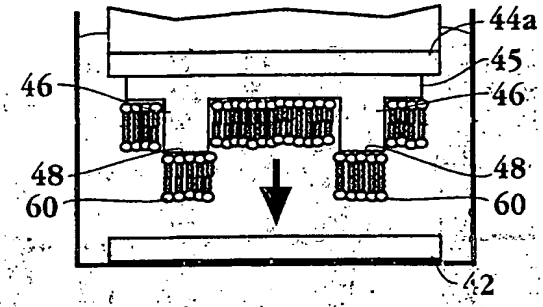

Fig. 2G

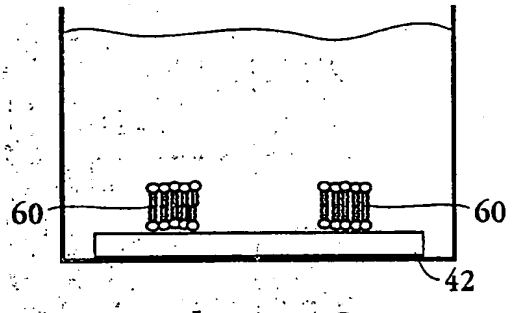

Fig. 2H

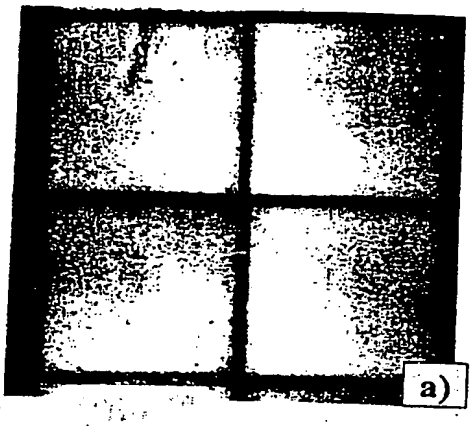

Fig. 3A

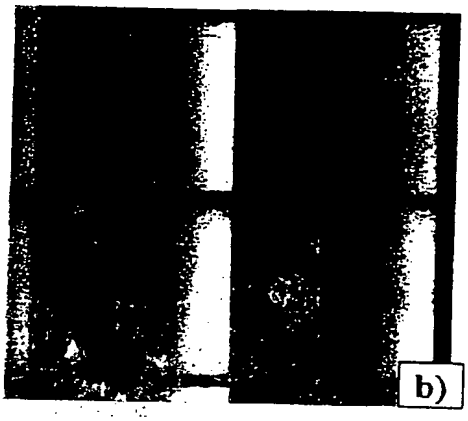

Fig. 3B

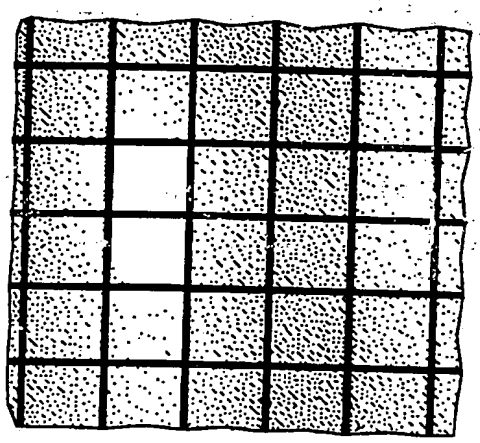

Fig. 3C

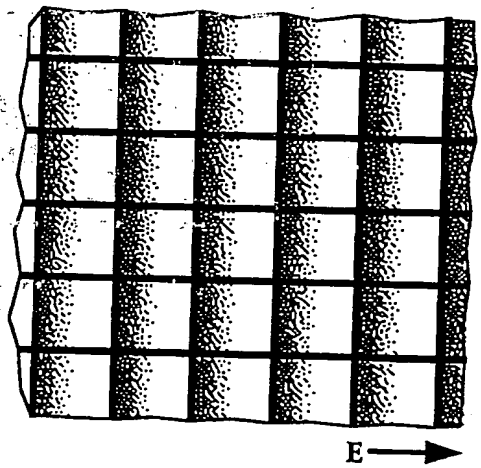

Fig. 3D

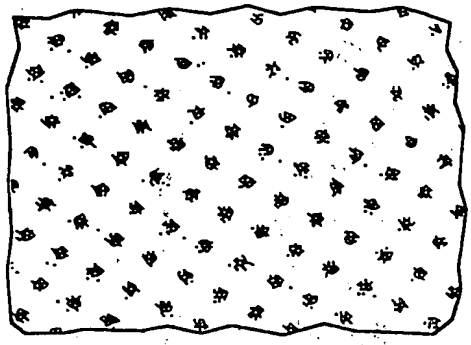

Fig. 3E

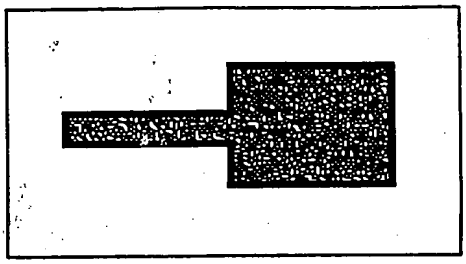

Fig. 3G

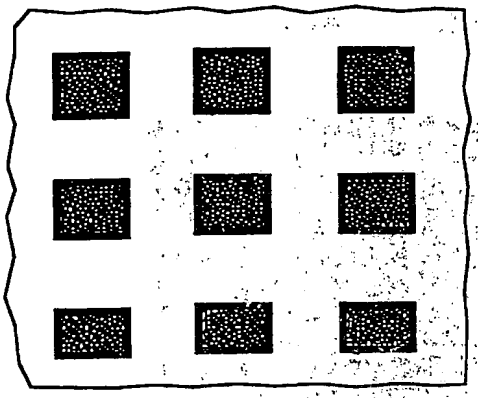

Fig. 3F

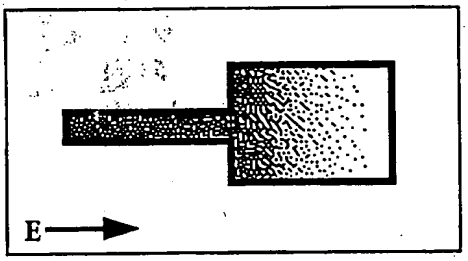

Fig. 3H

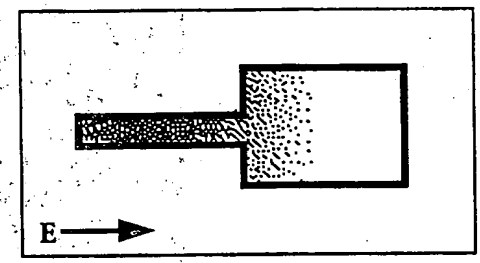

Fig. 3I

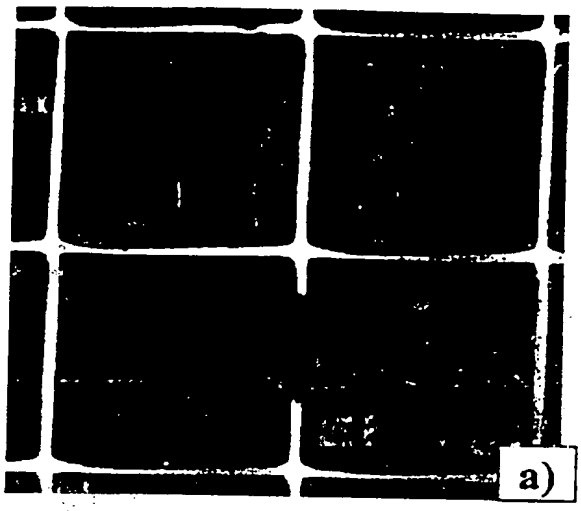

Fig. 4A

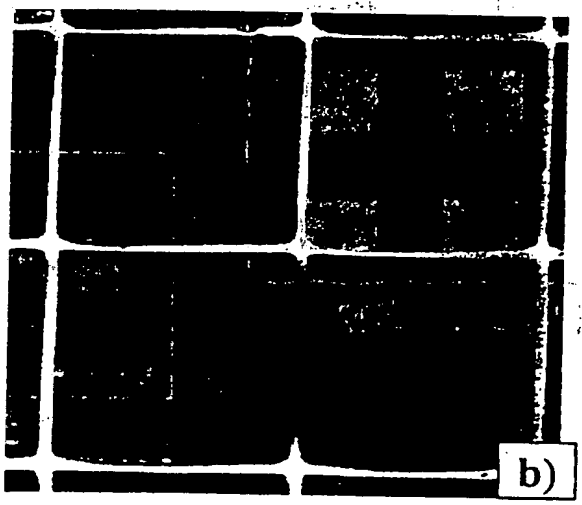

Fig. 4B

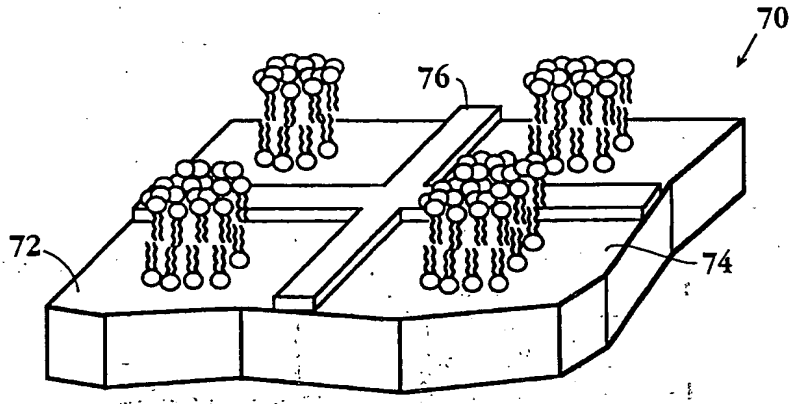

Fig. 5A

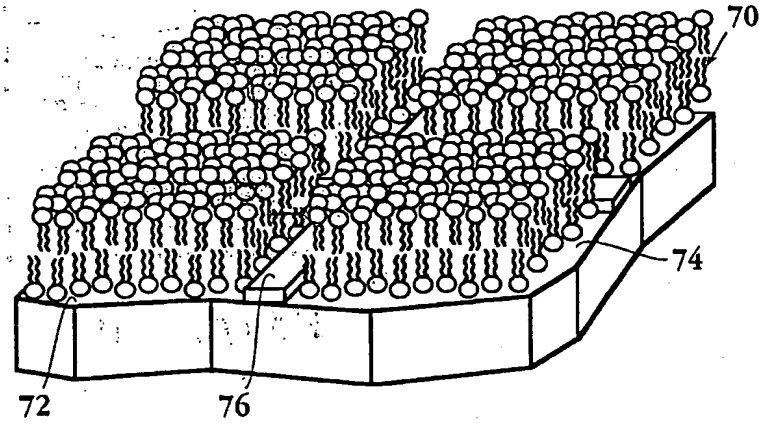

Fig. 5B

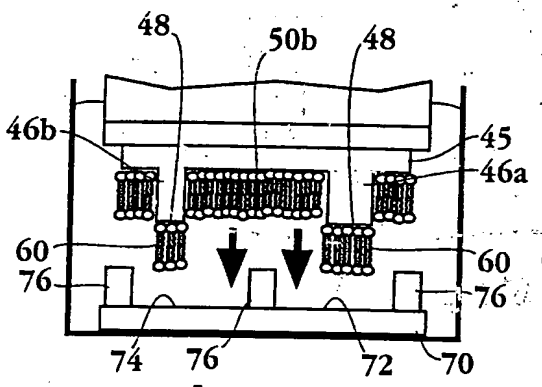

Fig. 6A

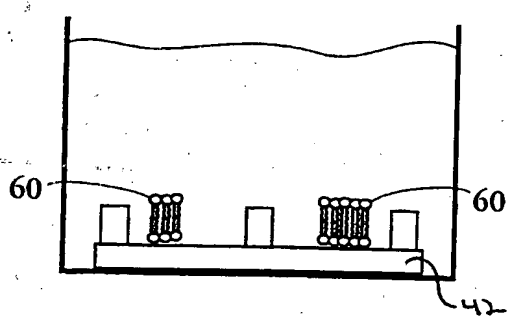

Fig. 6B

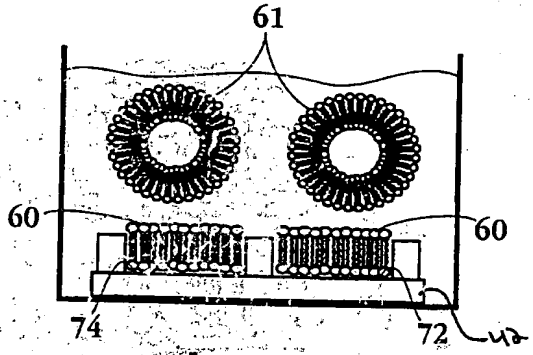

Fig. 6C

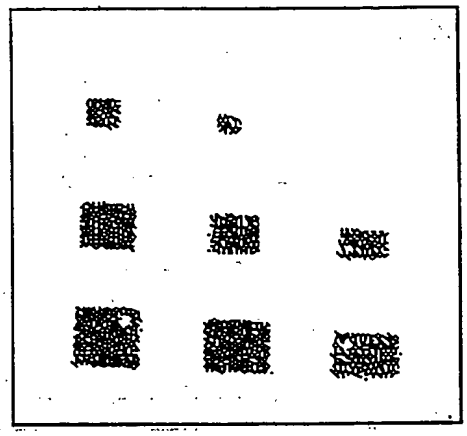

Fig. 7A

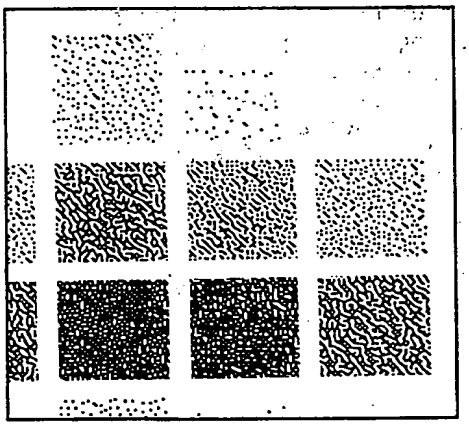

Fig. 7B

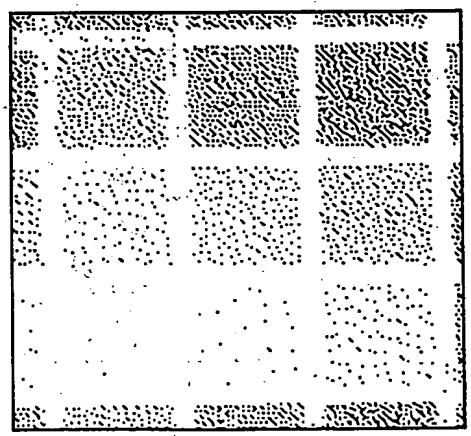

Fig. 7C

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 00/27774

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01J19/00 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 B01J G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 23948 A (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 4 June 1998 (1998-06-04) page 4, line 13 -page 5, line 25 page 7, line 18 - line 22 page 22, line 29 -page 23, line 26 figure 1	9-20
A	--- -/---	1-8, 21-23

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2001	08/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stevnsborg, N

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